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Protein quality control in the secretory pathway of eukaryotic cells

Kontrola kvality proteinů v sekreční dráze eukaryotních buněk

Bachelor's Thesis

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, dne

Markéta Bařinková

Abstract

More than 30 % of the cellular proteome enters the secretory pathway during biogenesis in eukaryotic cells. The secretory pathway then ensures that these proteins are correctly folded, undergo necessary post-translational modifications, and reach their target site in membrane organelles or outside of the cell. Since a significant number of the nascent proteins in the pathway are or become dysfunctional, the cell must possess quality control mechanisms by which to weed them out. As proteins travel through the secretory pathway they may be degraded by various pathways in the endoplasmic reticulum, Golgi apparatus, endosomes, or at the plasma membrane. These degradatory pathways utilize a number of molecules including chaperones, ubiquitin ligases, and many others. They are coordinated by a unifying principle – the unfolded protein response, which acts as a support mechanism in case the degradation pathways are overwhelmed. The study of protein quality control mechanisms is necessary as they help us understand the production of a significant portion of the cellular proteome. Furthermore, defects in these degradation pathways are linked to several human diseases such as cystic fibrosis or some neurodegenerative diseases. These protein degradation pathways have been studied for decades, but thanks to newer technologies, novel facts about this cellular machinery are still emerging.

Keywords: endoplasmic reticulum-associated degradation, ER-phagy, endosome and Golgi-associated degradation, plasma membrane quality control, unfolded protein response, proteostasis, protein degradation

Abstrakt

Více než 30 % buněčného proteomu vstupuje během biogeneze do sekreční dráhy v eukaryotických buňkách. Sekreční dráha pak zajišťuje, že jsou tyto proteiny správně složeny, projdou nutnými posttranslačními úpravami a jsou dopraveny ke svému cílovému umístění ať už v membránových organelách, nebo vně buňky. Protože ale značné množství proteinů vstupujících do této dráhy je nefunkční, nebo se nefunkční stane, musí buňka disponovat mechanismy pro kontrolu kvality proteinů, pomocí kterých je z buňky odstraňuje. Jak proteiny putují sekreční dráhou, mohou být degradovány několika způsoby jak v endoplasmatickém retikulu, tak v Golgiho aparátu, endosomech nebo na plasmatické membráně. V těchto drahách je využíváno mnoho molekul od chaperonů, přes ubikvitin ligázy a mnoho dalších. Jsou spojeny sjednocujícím principem, který se nazývá „unfolded protein response“ (reakce na nesložené proteiny). Ten tyto dráhy podporuje, pokud jsou přehlcené. Studium mechanismů kontroly kvality proteinů je nutností, neboť nám pomáhá osvětlit vznik značného množství buněčného proteomu. Poruchy v této dráze jsou navíc spojeny s řadou lidských onemocnění od cystické fibrózy po některé neurodegenerativní poruchy. Tyto degradační dráhy jsou zkoumány již několik desetiletí, ale díky posunům v technologii se stále vynořují nové informace o této mašinerii.

Klíčová slova: ERAD dráha, makroautofágie endoplasmatického retikula, EGAD dráha, kontrola kvality na plasmatické membráně, UPR dráha, proteostáze, degradace proteinů

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List of Abbreviations

AP	Autophagosome
ATZ	α1-antitrypsin Z
CBD	Canonical substrate-binding domain
CFTR	Cystic fibrosis transmembrane conductance regulator
CNX	Calnexin
CRT	Calreticulin
EMC	ER membrane protein complex
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERAD-C	ERAD of proteins with cytosolic lesions
ERAD-L	ERAD of proteins with luminal lesions
ERAD-M	ERAD of proteins with membrane-spanning lesions
ERAD-RA	Ribosome-associated ERAD
ERAD-T	Translocon-associated ERAD
ERAM	ER-to-autophagy membranes
ERES	ER-exit site
ERGIC	ER-Golgi intermediate compartment
ERLAD	ER-to-lysosome-associated degradation
GA	Golgi apparatus
Gls1	Glucosidase I
Gls2	Glucosidase II
GPI	Glycophosphatidylinositol
GPI-A	Glycophosphatidylinositol-anchored
HMGR	β-Hydroxy β-methylglutaryl-CoA receptor
LIR	LC3 interaction motif
MIDY	Mutant INS-gene-induced diabetes of youth
Mns1	α-mannosidase
MRH	Mannose-6-phosphate receptor homology
MVB	Multivesicular body
NAG	N-acetyglucosamine
PAS	Pre-autophagosomal structure
PDI	Protein disulfide isomerase
PI(3)P	Phosphoinositol-3-phosphate
PM	Plasma membrane
QC	Quality control
RER	Rough endoplasmic reticulum
RHD	Reticulon homology domain
RQC	Ribosome quality control
RTN3C	Short RTN3 isoform
RTN3-L	Long RTN3 isoform
SER	Smooth endoplasmic reticulum
SRP	Signal recognition particle
TA	Tail-anchored
TGN	Trans-Golgi network
TM	Transmembrane

1. Introduction

The secretory pathway is an organelle complex containing some of the biggest cell compartments in eukaryotes – the endoplasmic reticulum (ER), the Golgi apparatus (GA), and the plasma membrane (PM). All these components along with lysosomes are inter-connected through the trafficking of membrane vesicles, which act as cargo transporters. Occasionally, membrane organelles are connected directly via membrane contact sites, such as ER-PM contact sites that mediate lipid transport and other, still emerging functions. More than 30 % of all cellular proteins are transported to the ER during biosynthesis, where they undergo post-transcriptional modifications, folding, and targeting. This makes the secretory pathway a key point for maintaining cellular homeostasis, as any defects could have an adverse effect on a significant proportion of the cellular proteome. To this end, organisms require sophisticated mechanisms for rooting out and degrading those proteins which are misfolded or rendered otherwise dysfunctional. The misfolding of proteins can lead to their loss of function, which may cause an unnecessary buildup of impaired proteins in the cell, or gain of function, which are potentially toxic.

To forego such complications, eukaryotic cells possess several protein quality control (QC) mechanisms built into their secretory pathway. These checkpoints ensure that as few damaged proteins as possible continue to their target locations and also remove protein aggregates from the secretory pathway. It is estimated that over 30 % of newly synthesized proteins in mammalian cells are misfolded [1], which only underlines the importance of such a system. The first and best described of these QC mechanisms is ER-associated degradation (ERAD). It participates in protein quality control (degrading faulty proteins), but also helps modify levels of ‘healthy’ proteins as a physiological regulatory mechanism responding to cellular needs. Misfolded molecules that elude this line of defense can be weeded out by ER-to-lysosome-associated degradation (ERLAD), which includes ER-phagy, microautophagy, and vesicular transport, or ER-independent control mechanisms, present in the GA and endosomes and at the PM. Malfunctions in these quality control pathways may lead to a number of diseases ranging from Alzheimer’s disease [2] to cystic fibrosis [3].

2. Introduction into the secretory pathway

In this chapter, I shall discuss the basic anatomy and principles of function of the secretory pathway, and some of the processes linked to protein quality control. Among the discussed topics are the mechanisms by which proteins enter the ER, glycosylation, and protein folding.

2.1 The secretory pathway

The secretory pathway is a membrane organelle system present in all eukaryotic cells. It consists of the endoplasmic reticulum (ER), Golgi apparatus (GA), endosomes, lysosomes (vacuoles in yeast), and the plasma membrane (PM). Although these organelles are separate, proteins flow between them depending on their target location, posttranslational modifications, and degree of folding. For a schematic overview of the secretory pathway please see *Figure 1*.

The ER is composed of three distinguishable regions with different functions – the nuclear membrane, the rough endoplasmic reticulum (RER), and the smooth endoplasmic reticulum (SER). The nuclear membrane can be further divided into the inner and outer membrane, which are joined by curved membrane segments surrounding nuclear pores and the proteins that form these pores. While the inner membrane's main function is to organize the nucleus, the outer nuclear membrane morphs smoothly into the ER with which it shares a similar function and morphology. In proximity to the nuclear envelope is the RER, which is coated with ribosomal units and is the main site of protein synthesis and posttranscriptional modifications. The rough ER then continues onto the smooth ER to form one continuous membrane complex. The smooth ER is mainly the site for the trafficking of vesicles as well as lipid biosynthesis. Apart from the synthesis of lipids and proteins, the ER also plays an important role as the cell's calcium reservoir.

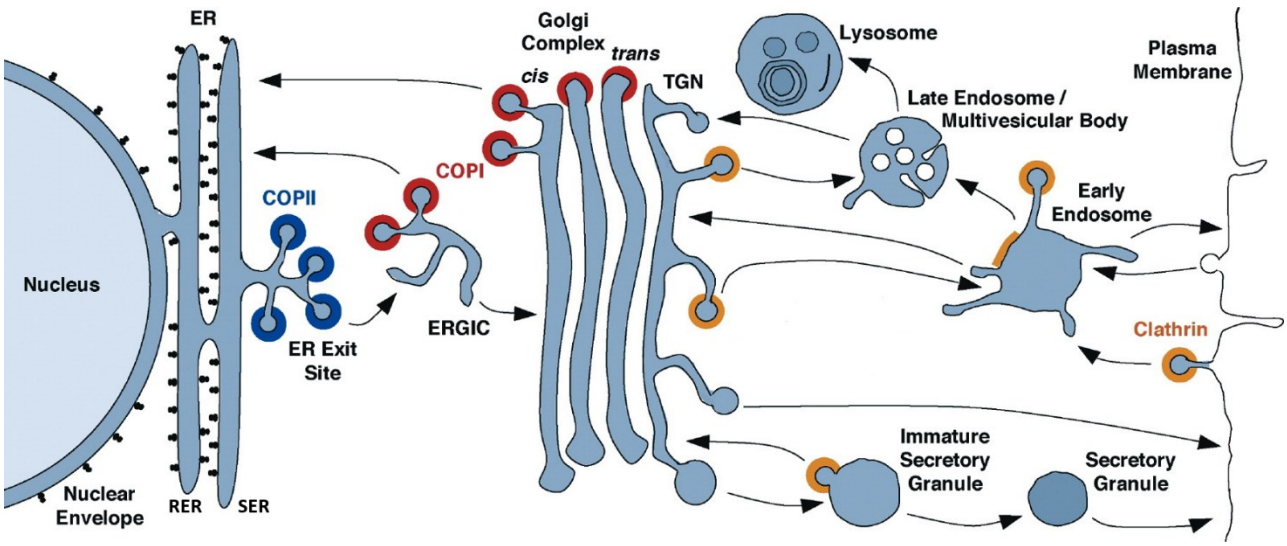

Figure 1 – Schematic depiction of the secretory and endosomal pathways in eukaryotic cells. Adapted from [146]. The directions of secretory pathway cargo movements are marked by arrows. Blue, red and orange colors indicate COPII-, COPI-, and clathrin-coated vesicles, respectively.

There are two basic morphologies of the endoplasmic reticulum – sheets and tubules. While sheets are formed by two membrane surfaces curved mainly at the edges, tubules resemble straw-like membrane

formations. Sheets are more common in the RER, while tubules are found mostly in the SER. ER sheets are stacked from the nuclear envelope outward and connected by helicoidal membrane structures [4].

From the ER, the secretory pathway continues onto the ER-Golgi intermediate compartment (ERGIC) via COPII-coated vesicles and then to the Golgi apparatus. This is formed by elongated cisternae which, unlike the sheets and tubules of the ER, are not directly interconnected. The cisternae display a *cis* face, which receives cargo vesicles and a *trans* face from which vesicles are trafficked to their final location. Vesicles from the *cis* faces can also be transported back to the ER via COPI-coated vesicles. As in the ER, proteins undergo posttranslational modifications in the lumen of the GA.

The outermost organelle of the secretory pathway is the plasma membrane. Proteins are trafficked here via secretory granules but may also be endocytosed into endosomes. These are then morphed into multivesicular bodies (MVBs). Here, vesicles containing proteins for degradation may also enter this pathway. MVBs are then degraded in a lysosomal manner.

2.2. Protein targeting and translocation into the secretory pathway

Proteins are translocated to the ER co- or post-translationally through the recognition of an N-terminal signal sequence by a signal recognition particle (SRP) [5] or molecular chaperones [6], respectively. During co-translational translocation, a translated stretch of hydrophobic amino acids is recognized by a signal-recognition particle. This complex is then recognized by an SRP receptor on the ER membrane. Both the SRP and its receptor dissociate from the ribosome and translated peptide soon, however, as the ribosome is handed over to a Sec61 translocon complex through which the protein is translated into the ER lumen. The translocation of the protein during translation is accompanied by GTP hydrolysis.

Other proteins are translocated to the ER post-translationally in an SPR-independent manner. This phenomenon has been studied mainly on tail-anchored (TA) or glycophosphatidylinositol-anchored (GPI-A) membrane proteins. The ER targeting of these proteins is reliant on cytosolic chaperones (such as Ssa1 or Ydj1) [7] or TRC40 (mammalian homolog of the yeast Get3) [8] recognizing their terminal sequences. Some TA proteins, typically those whose transmembrane segment has a relatively low hydrophobicity, are translocated using the ER membrane protein complex (EMC) [9]. GPI-A proteins seem to utilize a Sec61 translocon complex comprising Sec61, Sec63, Sec62, Sec71, and Sec72 molecules [10]. In addition, some polytopic membrane proteins, such as those with a poorly hydrophobic first transmembrane signal-anchor or internal transmembrane domains, such as some G-protein coupled receptors [11], or transporters [12], also require the auxiliary insertase and chaperone EMC mentioned above.

2.3. Posttranslational and post-translocational modifications in the secretory pathway

Inside the ER lumen, proteins undergo many posttranslational modifications. Among these are glycosylation, the attachment of the protein to the membrane by a glycophosphatidylinositol (GPI) anchor, or the formation of disulfide bonds stabilized by the lumen's oxidizing properties. The signal peptide sequence of co-translationally translocated proteins is also cleaved off by signal peptidase here.

2.3.1. Glycosylation

One of these posttranslational modifications and among the crucial steps for correct protein folding is the transfer of a well-conserved glycan [13] comprising 14 monosaccharide units – three glucoses (Glc), nine mannoses (Man) and two N-acetylglucosamines (NAG) as seen in *Figure 2* – from a membrane-anchored dolichol molecule to a recipient amido group of an asparagine side-chain (i.e., N-glycosylation). During protein folding in the ER the terminal glucose (Glc1) unit is cleaved off by glucosidase I, followed by the cleavage of the second glucose (Glc2) by glucosidase II. This allows the protein to enter calnexin (CNX) and calreticulin (CRT) cycles. In these cycles, glycoproteins undergo a series of de- and reglucosylations by glucosidase II and glucosyltransferase, respectively. Glucosyltransferase recognizes misfolded proteins and stops them from exiting CNX/CRT cycles. When a protein is properly folded, glucosidase II cleaves the third glucose, but the protein is not recognized for reglucosylation by the glucosyltransferase, which enables the deglucosylated glycoprotein to leave the cycle and continue onto its functional destination. The lingering of a glycoprotein in the ER for longer periods suggest that the molecule is irreparable. It may undergo the slower demannosylation by α-mannosidase. The absence of the central mannose residue (and therefore the activity of this mannosidase) was shown to have a significant effect on the rate of degradation [14, 15].

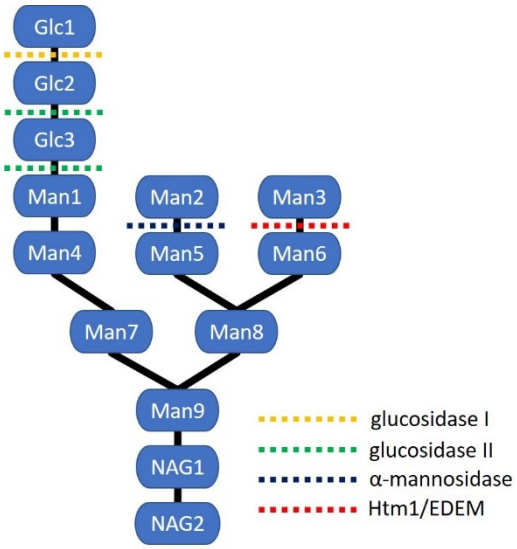

Figure 2 – A schematic representation of a $Glc_3Man_9GlcNAc_2$ molecule along with cleavage points (represented by dashed lines). Bonds between molecules are depicted by black lines. Glc – glucose; Man – mannose; NAG - N-acetylglucosamine.

In addition to this N-glycosylation, proteins may also undergo O-glycosylation both in the ER and GA. During this process, sugar molecules are bound to serine and threonine amino acid side-chains. A number of glycans may be connected including galactose, glucose, N-acetylgalactosamine, or N-acetylglucoseamine. These molecules help modify the activity and stability of proteins.

2.3.2. Disulfide isomerization and protein folding

Protein disulfide isomerases (PDIs) are an extensive protein group, which was first discovered as a "nonspecific catalyst for disulfide interchange in proteins containing disulfide bonds" [16] in the oxidizing environment of the ER. Their active sites include two cysteine molecules. They take part in redox reactions,

which are necessary for disulfide isomerization in proteins. Due to hydrophobic interactions, they interact more willingly with misfolded proteins [17]. In addition to PDIs, other chaperones such as BiP (Kar2 in yeast), calnexin and calreticulin help protein folding in the ER.

3. Endoplasmic reticulum-associated degradation (ERAD)

The most-studied protein quality control mechanism in the secretory pathway is ER-associated degradation (ERAD). Depending on the location of lesions caused by misfolded protein domains, three main pathways have been discovered in yeast – luminal (ERAD-L), cytosolic (ERAD-C), and membrane-spanning (ERAD-M). Of these, the most thoroughly studied is ERAD-L. These pathways can also overlap. In mammalian cells, the mechanism is more complex, probably due to the evolutionary distance and higher morphological and functional complexity and diversity of cells in higher multicellular eukaryotes. In addition to the mentioned three branches of ERAD, two ERAD-associated pathways are being studied, coined ribosome-associated ERAD (ERAD-RA) and translocon-associated ERAD (ERAD-T). These will only be discussed briefly, as information concerning them is scarce. As most publications on this subject are written regarding yeast (mostly *Saccharomyces cerevisiae*), I will focus mainly on this eukaryotic organism, though information will also be given concerning modifications present in higher eukaryotes. The ERAD mechanism can be broken down into four basic steps – the identification of substrates, ubiquitination, retrotranslocation, and degradation – which I will be examining throughout this chapter. For a schematic overview of ERAD pathways in yeast please see *Figure 3*.

3.1. ERAD-L

3.1.1. Substrate recognition

As was discussed in the previous chapter, one of the molecules significant for the degradation of misfolded proteins is a demannosylated glycan molecule. However, in some strains of yeast, this single demannosylation is shared by proteins which are both misfolded and targeted for the GA, showing that there must be other mechanisms for the detection of misfolded molecules. A lectin termed Htm1 (EDEM in mammals) was discovered as a specific mannosidase, cleaving Man3 from the $Man_8GlcNAc_2$ glycan and therefore uncovering an α-1,6-linked mannose [18]. The C-terminal domain of Htm1 was also found to form disulfide bonds with Pdi1 (mammalian homologs ERp57, ERp72 [19]), a protein disulfide isomerase. The failure to establish this complex leads to a significant decrease in protein degradation [20, 21], which points to the fact that Htm1 and, to an extent, a monodemannosylated glycol, is not enough to mark proteins for the ERAD pathway. As discussed in the introductory chapter, PDIs interact primarily with misfolded proteins, and Pdi1 could, therefore, ensure that Htm1 interacts specifically with proteins intended for degradation.

Misfolded glycoproteins marked by the α-1,6-mannose-presenting glycan are then recognized by a Yos9 molecule (OS9 and XTP3-B in mammals, [22]). This protein contains a mannose-6-phosphate receptor homology (MRH) domain, shown to be necessary for the degradation of proteins in the ER [23]. Given the available information, it would seem that and interaction between the newly exposed mannose molecule and

MRH domain would be a crucial step in protein recognition. It has been shown, however, that the initial Yos9 recruitment to the ERAD machinery is not entirely dependent on the presence of glycosylated amino acids and that this molecule can even recognize non-glycosylated proteins, which it protects from degradation [24]. Though Yos9 has the ability to attach to mannose molecules, this function is likely utilized later in the ERAD pathway as a checkpoint before retrotranslocation [25, 26]. The domain utilized in this primary misfolded protein recognition is still largely unknown.

The Yos9/misfolded protein complex then interacts with luminal Kar2 (BiP in mammals), an ER luminal Hsp70 (Heat-shock protein 70) variant, which increases the solubility of its substrates and therefore keeps them in the ER lumen and enables the recruitment of other chaperones. It has also been shown to decrease ERAD efficiency, which points to the fact that it may dissociate from the protein in the following steps [27].

This luminal complex is then delivered to the Hrd1 complex. The recruitment is mediated by Hrd3 (SEL1L in mammals), whose B subdomain has been shown to be crucial for Yos9-Kar2-glycoprotein complex attachment [25, 26]. Hrd3 was long thought to be a transmembrane (TM) molecule with a cytosolic tail, membrane anchor, and a sizable luminal domain. A recent study has shown, however, that Hrd3 may not act as a TM molecule in this complex at all, but rather as a luminal recognition particle for misfolded proteins [28]. The Hrd1 complex is formed by several proteins. Hrd1 (an E3 ubiquitin-protein ligase), Hrd3, Usa1 (HERP in mammals), and Der1 (DERLIN1-3 in mammals) form a unit, which is tightly bound, while Yos9, Ubx2 (a TM molecule needed for the recruitment of Cdc48, UbxD2/8 in mammals), and Cdc48 (p97 in mammals) seem to have a lower affinity to this “core complex” [25]. Usa1 has two transmembrane segments and both its terminal domains are located in the ER lumen. It acts as a scaffolding protein for Der1 and Hrd1 (which interact with the C-terminus and N-terminus of Usa1, respectively) as well as a helper in the oligomerization of Hrd1 [29, 30].

3.1.2. Ubiquitination

The Hrd1 ubiquitin ligase complex is the central system for the ubiquitination and retrotranslocation of ERAD-L substrates. For the ubiquitination of ERAD substrates to occur, at least two additional molecules of the ubiquitination complex must be recruited to the Hrd1 complex. Cue1 (mammalian homolog unknown) is a molecule containing the E3 ubiquitin ligase complex U7BR, which helps activate the Ubc7 E2 ubiquitin-conjugating enzyme (UBC7 in mammals) [31]. While Cue1 helps to tether Ubc7 to the Hrd1 complex, Ubc7 mediates the ubiquitination of the substrate. There is, however, also evidence to suggest that ubiquitination might not be necessary for the retrotranslocation of some substrates [32].

3.1.3. Retrotranslocation

There are two main theories as to how the substrates of ERAD-L are moved from inside the ER lumen to the cytosol. Some studies suggest the involvement of Sec61 [33, 34, 35, 36], a well-described protein channel active in the translocation of proteins into the ER. Others propose that the key retrotranslocon molecule is Hrd1 itself. This is supported by the fact that Hrd1, when overexpressed, can bypass the need for

other complex members such as Hrd3, Der1, or Usa1 (or indeed, all three together). This is probably due to the oligomerization that is usually facilitated by Usa1 in vivo but can happen spontaneously when Hrd1 is overexpressed [29]. The autoubiquitination of Hrd1 has also been shown to allow the retrotranslocation of substrates [32]. In 2017, the cryo-electron microscopy structure of Hrd1 in complex with Hrd3 was solved to show that five Hrd1 transmembrane domains form a cavity, while other domains form a gate. Together this leads to the formation of a structure similar to other translocons [37]. A 2020 study determined the structure of the Hrd1 complex with Der1, again using cryo-electron microscopy. Based on their findings a model was proposed in which a luminally based Yos9/Hrd3 complex recognizes a misfolded protein and moves it towards a two-part cavity formed by both Der1 and Hrd1. The formation of this cavity is enabled by the thinning of the plasmatic membrane by Hrd1 and Der1 alike. This way a loop forms from the misfolded protein presenting only a short chain to ubiquitin ligases in the cytosol for ubiquitination [28].

The driving force behind the retrotranslocation of ERAD substrates seems to be (at least in part) the ATPase function cytosolic Cdc48 molecule. Substrates are attached to Cdc48 via a K48-linked poly-ubiquitin chain on the substrate and the two Cdc48 cofactors (Ufd1 and Npl4, UFD1 and NPL4 in mammals). This allows for the molecule to be unfolded so that it can pass through the Cdc48 oligomer. The changes in interactions between all the factors lead to ATP hydrolysis, which in turn changes Cdc48 conformation and pulls at the unfolded substrate [38]. Polyubiquitin chains are then free to be accessed by the Otu1 deubiquitinase (mammalian homolog unknown). Substrate deubiquitination leads to the weakening of the substrate-cofactor interaction, release of the substrate and return of Cdc48 to its original conformation. After deubiquitination by Otu1, the ubiquitin chains are no longer long enough for proteasomal degradation of the substrate. Therefore, Ufd2 (and E4 ubiquitin-chain assembly factor, mammalian homolog UFD2 [39]) is associated with the Cdc48 C-terminus [40], and it elongates the chain by 3-6 ubiquitin elements, enabling the degradation of the misfolded protein substrate [41].

3.1.4. Degradation

The elongated ubiquitin chains act as a signal in the cytosol for proteasomal degradation. Early studies show that Dsk2 (mammalian PLIC1) and Rad23 (mammalian HR23A and HR23B) proteins take part in this process [42]. Rad23 was already known to have both ubiquitin and proteasome binding sites [43], which were later shown to help transport as much as 90 % of ERAD substrates to the 26S proteasome for degradation [44].

3.2. ERAD-M

The ERAD-M pathway may share some of the molecule complexes seen in ERAD-L, most importantly the Hrd1/Hrd3 complex [25], although its mechanism is not as well known. Other studies suggest that specific ERAD-M substrates may differ vastly in the mechanism through which they are recognized and translocated to the cytoplasm [45, 46, 47].

3.2.1. Substrate recognition

It has been shown on β-Hydroxy β-methylglutaryl-CoA reductase (HMGR) molecules that the TM domains of Hrd1 or of the Hrd1/Hrd3 complex itself may be able to recognize proteins misfolded in the

membrane region [48, 49]. These domains have been shown to interact with many transmembrane regions of not only misfolded proteins, so a further mechanism to target specifically misfolded proteins such as Ubc7 would be needed [48]. The high percentage of hydrophilic residues found on transmembrane domains of Hrd1 also support the hypothesis that some misfolded molecules could be directly recognized for degradation by this molecule [49]. Not all ERAD-M substrates are, however, selected for degradation by the Hrd1 machinery. Sbh2 has been shown to be an ERAD-M substrate recognized by the Doa10 molecule (an E3 ubiquitin ligase found mainly in the ERAD-C pathway, [47]). On the other hand, a misfolded XBP1 molecule is prone to signal peptide peptidase (SPP)-dependent degradation with Der1 and TRC8 cofactors [45]. Still more studies suggest that ERAD-M substrates such as αβTCR are translocated to the lumen of the ER when misfolded, where they are identified by BiP and marked for degradation [46].

3.2.2. Ubiquitination

The ubiquitination mechanism of ERAD-M seems to be identical to that of ERAD-L. It requires the recruitment of Cue1 and Ubc7, which attach ubiquitin chains to the target substrate. In addition to the proteins of the ERAD-L ubiquitination mechanism, ERAD-M contains deubiquitinases, which should ameliorate the effect of insufficient substrate recognition caused by the two-dimensional space of the membrane [50].

3.2.3. Retrotranslocation and degradation

Recent studies have shown that the derlin Dfm1 is needed for retrotranslocation in both the ERAD-M and ERAD-C pathways [51], though it had been previously disputed. Dfm1 also contains a cytosolic domain, which recruits the Cdc48 molecule [52]. This shows that Dfm1 has potential as a retrotranslocation molecule in ERAD. The exact mechanism by which this pathway would function is, however, unclear. Hrd1 and Dfm1 may cooperate in a way similar to Hrd1 and Der1 in ERAD-L. Both Der1 and Dfm1 are derlins, pseudoproteases of the rhomboid family [53]. The importance of this family of proteins in quality control mechanisms is evolutionarily conserved [54]. This was recently shown in bacteria, where, under the condition of transition metal stress, the YqgP rhomboid protease presents a polytopic transmembrane transporter of magnesium (MgtE) to the membrane-anchored AAA ATPase/protease FtsH, which enables the retrotranslocation and degradation of MgtE [55].

Cdc48 has also been shown as one of the more important molecules for the retrotranslocation of ERAD-M substrates. Its interaction with polyubiquitin chains on substrates renders the molecule soluble in the cytosol [56]. Degradation most likely follows the pattern of the ERAD-L mechanism.

3.3. ERAD-C

3.3.1. Substrate recognition

ERAD-C substrate molecules may be membrane-bound in either the ER or the cell nucleus. The E3 ubiquitin ligase Doa10 (MARCH6 in mammals), crucial for the ERAD-C pathway, was discovered to be resident in both the ER and nuclear envelopes. It has been suggested that the main recognition motif for Doa-mediated degradation is an uncovered amphipathic or hydrophobic sequence in the cytosol or nucleus [57]. It

seems that for the identification of ERAD-C substrates, heat-shock proteins are needed. The Hsp70 protein Ssa1 recognizes the substrate and requires two additional co-chaperones – Ydj1 and Hlj1 (HD2 and Hsp40 in mammals) – which are present to possibly enhance the interaction between the substrate and chaperone [58].

3.3.2. Ubiquitination

For correct ubiquitination, Doa10 requires both E2 ubiquitin ligases Ubc6 and Ubc7, where the latter forms a complex with Cue1 [59]. These molecules can also interact with the RING domain of Doa10. Ubc6 forms a bond between one ubiquitin unit and hydroxyl groups of amino acids and can, therefore, mark proteins for degradation even without accessible lysine molecules. These ubiquitin units are then elongated by Ubc7. All the above-mentioned steps are thought to be in place to prevent the ubiquitination of unimpaired proteins [60].

3.3.3. Retrotranslocation and degradation

Not much is known about the retrotranslocation of ERAD-C substrates. They may not need a retrotranslocation mechanism at all if they are associated with the ER membrane peripherally from the cytosolic side. If they have a transmembrane character, they can be translocated by the Doa10 molecule itself, which, similarly to Hrd1 might form a pore from its many transmembrane domains [57]. It has also been shown that Cdc48 is present in this Doa10 mechanism and most likely interacts with Dmf1 [51, 61], possibly to unravel the protein and mark it for proteasome degradation. Degradation is most likely to follow the pattern outlined in the ERAD-L mechanism.

3.4. ERAD-RA

ERAD-RA is a degradation pathway, which removes dysfunctional proteins that are stalled in the process of translation, i.e. even before they get fully translocated into the ER. This pathway has already been described as a ribosome quality control (RQC) pathway, functioning freely in the cytosol. Only a few studies have been done, however, on proteins during translocation to the ER lumen. With cytosolic proteins, the translated stretches exit the ribosome and can interact with factors soluble in the cytosol. If the ribosome is, however, already associated with the Sec61 channel for the translocation of a protein into the ER, these factors cannot reach the elongating peptide chain. It seems that, if the translation of the protein is stalled by the detection of an error, the protein can slide out of the Sec61 channel to such an extent, as to allow its targeting by RQC pathways [62].

An important factor in the ERAD-RA degradation is the Ltn1 (the yeast homolog of the mammalian LISTERIN) E3 ubiquitin ligase molecule [63]. An even more crucial molecule, however, appears to be Dom34, which is responsible for the dissociation of ribosomal subunits. It is hypothesized that this process allows the protein to leave the Sec61 channel either into the ER or cytosol, where it can be degraded [64].

3.5. ERAD-T

The final ERAD pathway I would like to mention is translocon-associated ERAD. This pathway specifically targets proteins, which are associated with translocon channels (such as Sec61) for abnormally

long periods. This can be caused by their inability to exit through them due to structural discrepancies, or due to an error in the channel itself. These proteins are shown to be sought out by the Hrd1 molecule discussed above, even if they are targeted by other mechanisms when not associated with the translocon [65].

3.6. Quality control at the nuclear membrane

Another specific QC pathway is located at the nuclear membrane, where misfolded substrates unable to leave the nucleus are targeted. Here three novel E3 ubiquitin ligases were found to cooperate with Ubc7 for the degradation of these proteins, Asi1, Asi2, and Asi3. These three molecules form the Asi complex which, along with a Cue1 tethered Ubc7 and associated Cdc48, removes misfolded proteins from the inner nuclear membrane [66, 67]. As discussed above, Doa10 is also present in the inner nuclear envelope and is also responsible for the quality control of its resident proteins.

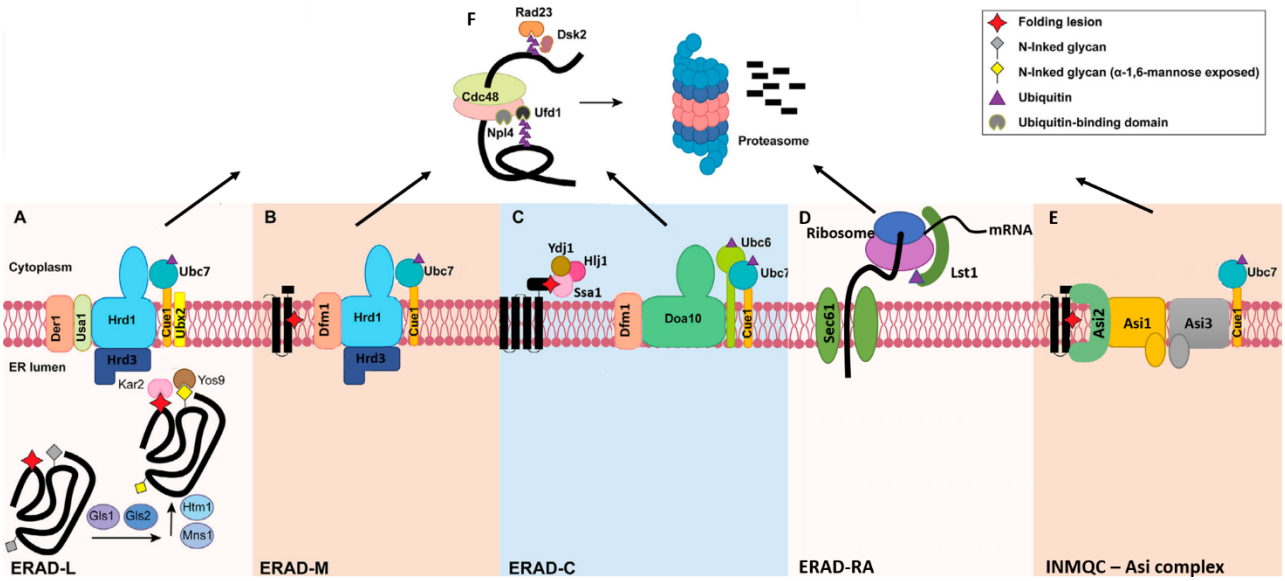

Figure 3 – Overview of ERAD pathways present in yeast. Adapted from [92]. **(A)** – ERAD-L substrates have luminal folding lesions (depicted as red stars). They contain N-linked glycans (shown as grey diamonds), which are shortened by glucosidase I, glucosidase II, α-mannosidase and Htm1 to expose an α-1,6-linked mannose (shown as yellow diamonds). The folding lesion is recognized by Kar2, while the glycan interacts with Yos9. This complex is then recognized by the Hrd1 membrane complex, which mediates its retrotranslocation and ubiquitination (depicted by a purple triangle). **(B)** – ERAD-M substrates have folding lesions located inside the membrane and are recognized, retrotranslocated and ubiquitinated directly by the Hrd1 complex. **(C)** – ERAD-C substrates have folding lesions in the cytosol that are recognized by Ssa1, Ydj1 and Hlj1 chaperones, which help their interaction with the Doa10 complex. This then mediates substrate retrotranslocation and ubiquitination. **(D)** – ERAD-RA targets proteins while they are being co-translationally translocated into the ER via a Sec61 translocon. Lesions in the translated protein cause translation to stop and enable the recognition and ubiquitination of this protein by Lst1. **(E)** – Misfolded proteins in the inner nuclear membrane may be removed from the ER and ubiquitinated by the Doa10 complex described in **C** or by the Asi complex shown in **E**. **(F)** – The retrotranslocation of all these proteins is aided by Cdc48, which marks them for degradation. Rad23 and Dsk2 then transport ERAD substrates to the proteasome for degradation. For references please see text.

3.7. Mammals

The mammalian ERAD pathway seems to be less straightforward than its yeast counterpart. Many more E3 ubiquitin ligases are known [68] and their functions may overlap in different pathways (as reviewed by [69]). In a recent study, Fenech et al. subjected mammalian ERAD interactomes to an extensive mapping which revealed interactions of 21 mammalian E3 ubiquitin ligase molecules [68]. The overall mechanism is, however, similar to that of yeast. First, ERAD substrates are recognized by chaperones and lectins, which transport the substrates to a ubiquitination and retrotranslocation complex in the ER membrane, where the

misfolded proteins are transported to the cytosol via an ATP-dependent mechanism using the p97 ATPase. From here the proteins are targeted to the proteasome for degradation. For an overview of the proteins involved in the mammalian ERAD pathway and their yeast homologs, see **Table *1*** below.

Yeast	Mammals	Function
Cdc48	p97	Substrate retrotranslocation
Cue1	Unknown	Ubc7 recruitment
Der1/Dfm1	DERLIN1, DERLIN2, DERLIN3	Retrotranslocation
Doa10	MARCH6	Ubiquitination and retrotranslocation
Dsk2	PLIC1	Transport to the proteasome
Hlj1	Hsp40	Ssa1 co-chaperone
Hrd1	HRD1, gp78, etc. (for more see [69])	Ubiquitination and retrotranslocation
Hrd3	SEL1L	Hrd1 stability
Htm1	EDEM1, EDEM2, EDEM3	Glycan trimming
Kar2	BiP	Glycan binding
Npl4	NPL4	Substrate recruitment to the Cdc48 complex
Otu1	Unknown	Deubiquitination, Cdc48 complex member
Pdi1	ERp57, ERp72	ERAD substrate interaction
Rad23	HR23A, HR23B	Transport to the proteasome
Ssa1	Hsp70	Substrate recognition
Ubc6	Ube2j1, Ube2j2	Ubiquitin conjugation
Ubc7	Ubc7	Ubiquitin conjugation
Ubx2	UbxD8, UbxD2	Cdc48 recruitment
Ufd1	UFD1	Polyubiquitin binding, Cdc48 complex member
Ufd2	UFD2	Ubiquitination, Cdc48 complex member
Usa1	HERP	Scaffold for Hrd1 and Der1
Ydj1	HDJ2	Ssa1 co-chaperone
Yos9	OS-9, XTP-3B	Glycan binding

Table 1 – Table of proteins involved in yeast ERAD along with their basic functions and mammalian counterparts. For references please see text.

4. ER-to-lysosome associated degradation (ERLAD)

Although it may seem that most misfolded proteins should be degradable by the ERAD machinery, some proteins still elude this quality control mechanism. This could be due to the inability of the mechanisms discussed above to recognize the misfolded proteins, the size of degradation substrates, or their tendency to form aggregates. As shown on the example of procollagen, some proteins also do not present hydrophobic residues in the ER lumen and are not, therefore, caught by the chaperones of ERAD. There is another mechanism for the removal of these molecules right in the ER, which has recently been termed ERLAD (ER-to-lysosome associated degradation) as it uses lysosomes (vacuoles in yeast) as its primary garbage can as opposed to ERAD, which uses proteasomes for degradation. ERLAD is mediateds by three distinct disposal mechanisms – ER-phagy (macroautophagy), ERES microautophagy, and vesicular transport – all of which

will be discussed in the following chapter. For a schematic overview of ERLAD pathways please see *Figure 4*.

4.1. ER-phagy

ER-phagy is the macroautophagy of ER regions. ER cargo targeted for degradation is transported via autophagosomes to the lysosome (vacuole in yeast). The ER-phagy pathway requires the cellular autophagy apparatus for its correct function. It is one of the mechanisms through which cells survive under ER-stress or starvation and is, in these cases, non-specific. There are, however, molecules that can also mediate a specific macroautophagy response to dispose of specific damaged or aggregated molecules. It is unknown, whether the specific autophagy as a pathway of quality-control in the ER uses the same mechanisms as autophagy mediated by ER-stress.

The formation of autophagosomes and their subsequent incorporation into the vacuole consists of several steps, some of which are better described for ER-phagy than others. The mechanism by which the cell recognizes cargo for vacuolar degradation, for example, is still not known. According to Lipatova et al., macro-ER-phagy comprises three separate steps – the formation of ER-to-autophagy-membranes (ERAM), the formation of the pre-autophagosomal structure (PAS) and lastly, the formation of autophagosomes (APs) [70].

Some studies suggest that COPII coated vesicles from ERGIC (ER-Golgi intermediate compartments) are also substrates for autophagy upon cell starvation. Its role in protein quality pathways is yet to be determined [71].

4.1.1. Autophagy mechanism

The first step of autophagy is the formation of ER-to-autophagy membranes (ERAMs). Single-membrane Atg9 vesicles formed from the GA fuse to form the ERAM, which later expands to form the double-membrane autophagosome [72]. These membranes contain Atg8 (LC3 in mammals) molecules, by which they interact specifically with ER segments containing macrophagy cargo. Though the exact mechanism of cargo recognition is not well described, it is known that Atg11, the prephagosomal structure organizer, is crucial. It recruits Ypt1 (a GTPase, hRab1 in humans) and TRAPIII (a guanine nucleotide exchange factor) to the cargo [73, 74]. On the cargo membrane, Atg11 also interacts with Atg39 and Atg40, which target the cargo specifically to the autophagosome via association with Atg8 molecules on ERAMs. Atg39 is specific for nuclear ER degradation, while Atg40 is thought to have a similar function as FAM134B in mammals (see *Chapter 4.1.2.1.* for more information) [75]. Atg11 further recruits more Atg9 vesicles to the cargo, which leads to the broadening of the phagosomal structure. A specific member of the TRAPPIII complex – Trs85 – then activates Ypt1 [73, 74]. ERAMs encircle the autophagy cargo leading to the formation of a double-membrane enclosed autophagosome in whose outer membrane the Atg9 molecules are localized. Atg1 (Ulk1 in mammals) is then recruited to the cargo and its interaction with Atg13 on the vacuolar membrane [76], along with Atg11 action recruits the cargo to the vacuole. The colocalization of Atg1, Atg11 and Atg13 leads to the activation of the kinase activity of Atg1 [77]. Ypt51 (Rab5 in mammals) is then responsible for the fusion of the autophagosome with the vacuole.

Cui et al. have also demonstrated that one of the differences between macroautophagy vesicles and ER-to-Golgi vesicles is the presence of Sec24 on the latter mentioned [78]. It has also been shown that Lst1 (the yeast homolog of the mammalian SEC24C) is also a necessary cofactor for ER-phagy, as it interacts with Atg40 and helps with the localization of the ER into autophagosomes [78]. Another important factor is Lnp1, which stabilizes three-way junctions in the native ER but also in the places of ER-phagy vesicle formation [79].

4.1.2. ER-phagy in mammals

In humans, two main ER-phagy pathways seem to operate – FAM134B-mediated and RTN3-mediated autophagy, although other autophagy receptors are also known (CCPG1, SEC62, ATL3 or TEX265). Both these pathways have been shown to need SEC24C for lysosomal degradation [78].

4.1.2.1. FAM134B-associated ER-phagy

FAM134B is one of the best-described autophagy molecules in humans. It is primarily localized in ER sheets, where it can induce autophagy under certain conditions. It is still unclear, how the FAM134B machinery is able to distinguish between membranes ripe for degradation and those functional and crucial to the ER function. In recent years, there has been a number of studies on the role of the FAM134B molecule in this process. FAM134B is an integral membrane protein containing a reticulon homology domain (RHD) and an LC3 interaction motif (LIR), which enables the molding of the ER membrane and interaction with LC3 molecules found in the membranes of autophagosomes, respectively [80, 81]. It has been shown to form oligomers during ER-phagy mediated by the phosphorylation of a serine residue by CAMK2B, which leads to the fragmentation of the ER membrane [82]. CAMK2B is a calcium/calmodulin-dependent protein kinase. Its function is stimulated therefore, by increased intracellular calcium concentration. This reflects the fact that autophagy is not only a housekeeping mechanism but also a stress-response pathway.

Apart from the autophagy of aggregated membrane proteins, it has also been shown that, through cofactors, ER-phagy can specifically target luminal-ER proteins, whose size restricts them from being degraded via ERAD. One such example is procollagen. In this particular case, FAM134B is necessary for degradation, but as it has no luminal domain, another protein is necessary for the recognition and degradation of misfolded procollagen. Forrester et al. showed that the molecule is in this case the chaperone calnexin, which forms a bridge between misfolded procollagen and FAM134B [83]. Calnexin is an important protein in ER quality control pathways as described above. This raises the question of whether other molecules could be targeted by a complex formed by calnexin and FAM134B. As was shown in this study, however, the main proteins which are disposed of through this pathway are indeed collagen molecules. It is however possible that other chaperones can mediate this response for other ER-phagy substrates [83].

4.1.2.2. RNT3-associated ER-phagy

Unlike FAM134B, RTN3 is mainly found in tubular sections of the ER and is found in two isoforms in cells (long and short). These two isoforms of RTN3 differ in the much longer N-terminal domain of RTN3-L, which is required for the interaction with LC3 by six LIR motifs. It also contains an RHD domain. As with

FAM134B, the main force behind the fragmentation of the ER is the aggregation of RHD domains of RTN3, which leads to the bending of the ER membrane and its eventual disconnection from the ER organelle. The ER fragments are then enveloped by the autophagosome and removed to the lysosome base on the LIR-LC3 interaction [84]. Surprisingly, Cunningham et al. showed that ER-phagy could be induced in *RTN3-L* knockout-cells through interactions of RTN3C (the short RTN3 isoform) or RTN4A with LC3, though neither contains LIR motifs [85].

The RTN3-associated ER-phagy mechanism is well described on cells with mutations leading to the aggregation of proinsulin in the ER and MIDY (mutant INS-gene-induced diabetes of youth) in humans. It is, however, a degradation pathway for other proteins such as proopiomelanocortin and pre-arginine vasopressin [85].

4.2. ERES microautophagy

In contrast to ER-phagy, microautophagy does not require the formation of an autophagosome. Instead, ER-exit site (ERES) regions are invaginated and directly enveloped by lysosomes/vacuoles for degradation. As shown by Omari et al., microautophagy may be another mechanism of misfolded procollagen clearing [86]. In their study, misfolded procollagen filaments accumulated in ER-exit sites and formed vesicles coated by COPII and autophagy-related molecules. In contrast to ER-to-Golgi vesicles, these autophagy vesicles were quite stable at ERESs, while vesicles trafficking proteins to the GA left the ER dynamically. The vesicles for degradation were then enveloped by nearby invaginated lysosomes. Though it is unclear exactly how these sites are marked for degradation, it is suggested that the surface proteins may be tagged for degradation by ubiquitin molecules [86].

4.3. Vesicular transport

The final proteasome-independent ER protein degradation pathway is vesicular transport. To this day it has been described on the single example of α1-antitrypsin Z (ATZ) polymers resistant to proteasome degradation. In this pathway, vesicles separated from the ER travel through the cytoplasm and to lysosomes, where they are engulfed. Calnexin molecules are thought to act as substrate receptors, which form a complex with FAM134B in the ER membrane. FAM134B forms oligomers, which causes the formation of an independent vesicle. This vesicle is then transported to the lysosome and connected via a FAM134B-LC3 interaction. The fusion of the membranes is realized by ER and lysosome SNAREs (STX17 and VAMP8, respectively) [87].

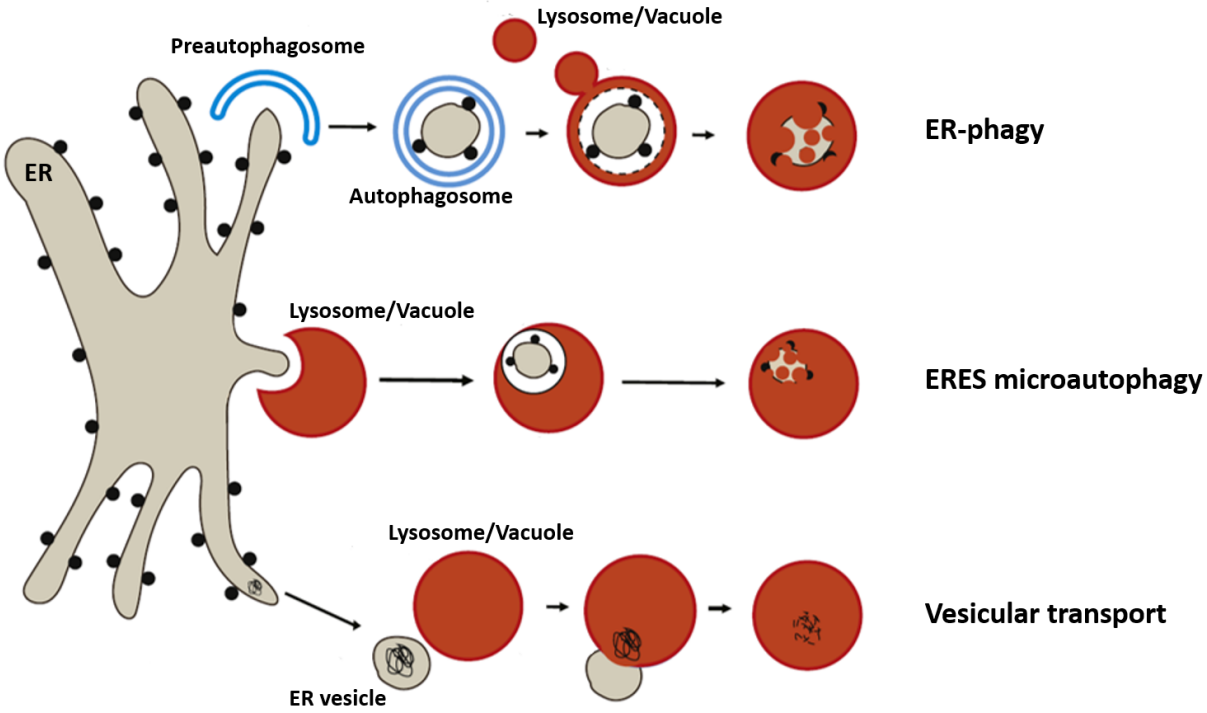

Figure 4 – Overview of ERLAD pathways. Adapted from [145]. The ERLAD mechanism utilizes three separate degradation mechanisms**. ER-phagy** is the macroautophagy of ER regions. Segments of the ER containing degradation substrates are enveloped by double/membrane autophagosomes which deliver them to lysosomes/vacuoles for degradation. **ERES microautophagy** is a mechanism independent of autophagosome formation. In this mechanism, ER segments targeted for degradation are enveloped directly by the invagination of a nearby vacuole/lysosome. The final ERLAD mechanism is **vesicular transport**, during which vesicles containing ERLAD substrates bud from the ER and travel to lysosomes/vacuoles for degradation. For references please see text.

5. Endosome and Golgi associated degradation (EGAD)

Despite the existence of the pathways described above to rid the cell of misfolded or otherwise damaged proteins directly in the ER, there are still molecules, which cannot be degraded while passing through the ER. It has also been shown that proteins with strong ER-exit signals may be ignored by the ER quality control machinery a trafficked speedily to the GA [88, 89]. These proteins may then be removed by the so-called EGAD – Endosome and Golgi-associated degradation. For a schematic overview of EGAD pathways please see *Figure 5*.

One of the mechanisms by which the cell can secure that misfolded proteins do not continue in the secretory pathway is to transport them back into the ER for another attempt at ER(L)AD. It has been shown that proteins with hydrophobic amino acid residues forming target sequences in their TM domains that have not been targeted for degradation in the ER are transported back via an Rer1-mediated pathway (in mammalian cell) [90]. In yeast cells, this function is carried out by the orthologue Rer1, which is localized in cis-Golgi where it recognizes transmembrane domains of proteins targeted for the ER [91].

Mechanisms that remove misfolded proteins straight from the GA for degradation are divided into two basic groups (as proposed by Sun & Brodsky) based on the place of degradation – proteasome- and

vacuole/lysosome-targeted degradation [92]. The latter can be further categorized into receptor- or ubiquitin-mediated Golgi quality control.

5.1. Proteasome-targeted

One of the proteins on which EGAD is studied is Orm2, which is degraded solely by means of this pathway for reasons which have not yet been clarified. The mechanism by which EGAD pathways recognize substrates for degradation also remains a mystery. Upon transport to the GA (mediated by TORC2-Ypk1-dependent phosphorylation), misfolded proteins are recognized and transported to the Dsc protein complex in the membrane of the GA. It seems that the phosphorylation from ER-export is an important signal for degradation by the EGAD machinery. Dsc comprises the E3 ubiquitin ligase Tul1 (which is tethered to the GA by Gld1, mammalian homologs are still unknown), Ubx3 (which, similar to Ubx2 in the ERAD pathway recruits Cdc48, mammalian counterpart UBXD8) and Dsc2 (a ubiquitin ligase similar to Der1 in the ERAD pathway, mammalian orthologue UBAC2) [93]. Both Dsc2 and UBAC2 are pseudoprotease members of the rhomboid family, which was mentioned in *Chapter 3.2.3*. Orm2 is ubiquitinated by this complex at K25 and K33. As in some of the above-mentioned ERAD pathways, Cdc48 plays an important role in the extraction of EGAD substrates from the membrane. After this, the protein is targeted for proteasomal degradation. It is also noteworthy that a similar mechanism is active in endosomes [94]. The ubiquitination in these steps can, however, also lead to an ESCRT (endosomal sorting complex required for transport) pathway leading to vacuolar degradation as described in *Chapter 5.2.2.* and shown in *Figure 6*.

Recently, it has been shown in mammalian cells that native Golgi proteins are degraded by a membrane-bound proteasome following cellular stress. The study shows a mechanism by which a membrane-bound proteasomal unit along with p97 (the mammalian homolog of Cdc48) helps in degrading Golgi proteins and suggests that a similar pathway may be involved in post-ER protein quality control in mammals [95]. A homolog of the Tul1 complex has also been found in *Saccharomyces pombe*, which seems to target and Golgi proteins for proteasomal degradation by an Hrd1-like mechanism. The Tul1 homolog Dsc1 forms a complex similar to the Hrd1 complex in ERAD [96]. Further studies showed the interaction of this complex with Dsc5, a protein containing a UBX domain, along with Cdc48 [97]. Not many other proteasome-targeted degradation pathways in the GA have been described, but from this example, it would seem that the ERAD and proteasome-targeted EGAD share similar pathways, at least for degradation.

5.2. Vacuole/lysosome-targeted degradation

5.2.1. Receptor-mediated

One of the receptor molecules recognized as a vacuolar-targeting protein is Vps10. In yeast, this protein has been shown to target native vacuolar proteins from the Golgi complex to the target organelle based on a variety of motifs. As its deletion from cells causes misfolded proteins to travel onto the plasmatic membrane, it is shown to be a crucial protein for quality control [98]. Though it has been shown that the cytoplasmic tail of Vps10 is necessary for vacuolar sorting [99], not much else is known about the interactions

of this receptor with its substrates, apart from the fact that the process is saturable by high levels of misfolded proteins [98].

In mammals, a family of proteins containing a Vps10 domain was discovered. Among these is sortilin, which was shown to specifically recognize aggregated GPP130 molecules targeted for the lysosome of mammalian cells [100].

5.2.2. Ubiquitin ligase-mediated

The ubiquitin ligase-mediated degradation of misfolded proteins in the GA is a process dependent on the formation of multivesicular bodies (MVBs) via the ERCRT mechanism. The ubiquitination of proteins marked for degradation takes place after the trafficking of proteins from the ER to the GA. Ubiquitination may be mediated by one of two ubiquitin ligases – Rsp5 and the above-mentioned Tul1.

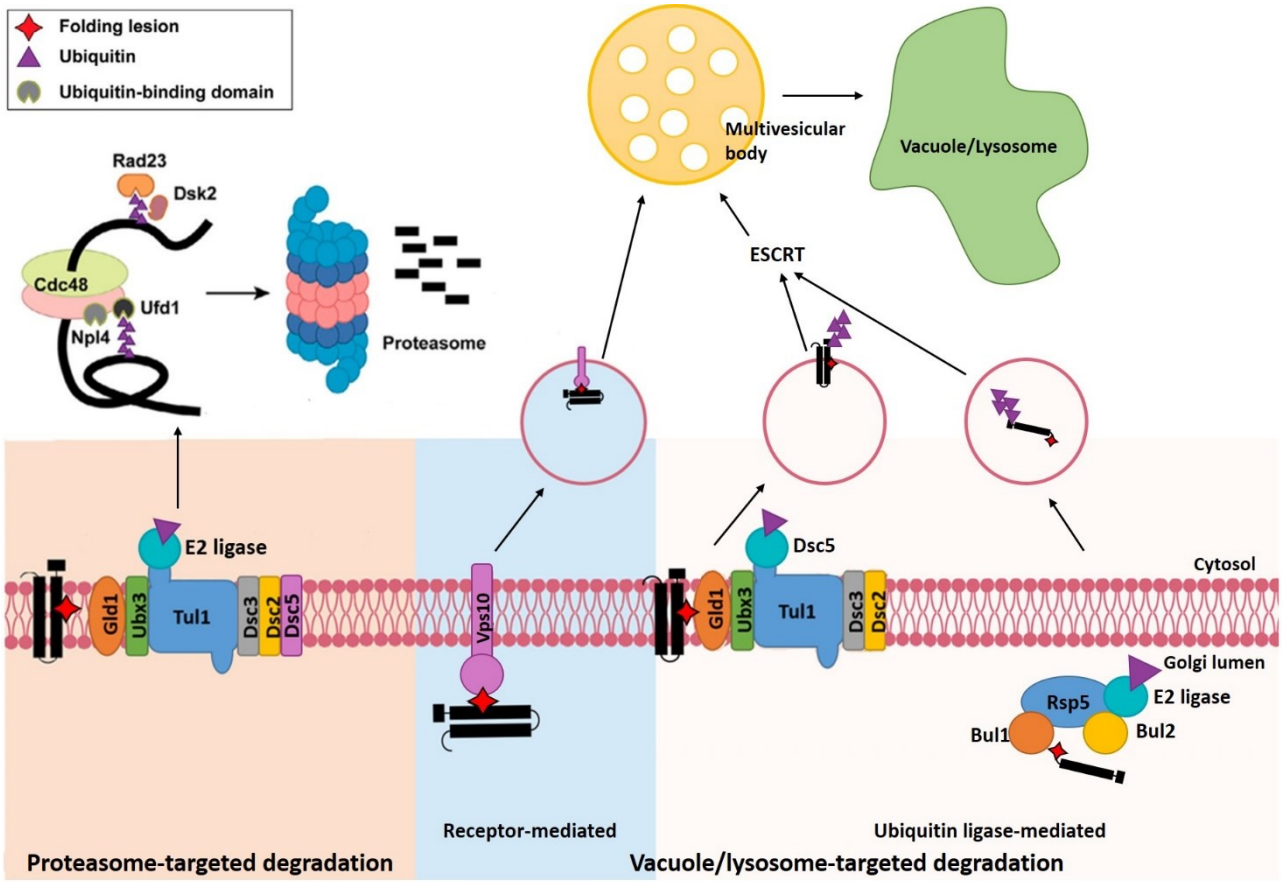

Figure 5 – Overview of EGAD pathways of eukaryotic cells. Adapted from [92]. EGAD is divided into two main groups – proteasome- and vacole/lysosome-targeted degradation. **Proteasome-targeted degradation** utilizes a Tul1 protein complex for the recognition of protein lesions (marked by red stars) and the retrotranslocation and ubiquitination (purple triangle) of these misfolded proteins. For retrotranslocation and the unravelling of the protein the Cdc48 complex is used and a combination of Rad23 and Dsk2 transport the substrate to the proteasome for degradation. **Vacuole/lysosome-targeted degradation** can be either receptor- or ubiquitin ligase-mediated. **Receptor mediated** degradation includes specific receptors (such as Vps10) for folding lesions. The interaction between the receptor and substrate leads to the formation of a vesicle, which is later transported to the lysosome for degradation. **Ubiquitin ligase-mediated** degradation may also utilize the Tul1 complex. Another complex recognizing protein lesions in an Rsp5 complex. Both the complexes in this pathway do not, however, recruit Cdc48 and ubiquitinated proteins leave the ER in vesicles. These are then transformed into MVBs via the ESCRT pathway and are degraded in the vacuole/lysosome. For references please see text.

Ubiquitination mediated by the E3 ubiquitin ligase Rsp5 requires ubiquitin ligase-binding proteins Bul1 and Bul2 [101]. The C2 domain of Rsp5 is responsible for cargo ubiquitination, as well as interacting

with phosphoinositides such as PI(3)P (phosphoinositol 3-phosphate), which can be a signal for the recruitment of Fab1, a kinase necessary for the sorting of proteins into MVBs [102, 103] located on the ESCRT-0 complex.

Tul1-mediated ubiquitination is carried out by the Tul1 E3 ligase complex comprising Tul1, Dsc2, Dsc3, and Ubx3 in *Saccharomyces cerevisiae*. Tul1 interacts with the formed Dsc2-Dsc3-Ubx3 subcomplex and helps sort substrates to MVB. It also recruits Ubc4 for the ubiquitination of substrates at its cytosolic (RING) domain [104]. The ways in which this pathway differs from the proteasome-mediated one is not yet known, though it is possible that the absence of Dsc5 could lead to the initiation of a vacuole-targeted pathway [104].

Ubiquitinated substrates are recognized by the ESCRT-0 complex formed by Vps27 and Hse1 in yeast (Hrs and STAM in mammals). Though Vps27 and Hse1 are similar, Vps27 contains an additional FYVE domain, which interacts with a phosphoinositol 3-phosphate (PI(3)P) on membranes via its Fab1 part [105]. Both molecules can also interact with ubiquitin. This combination allows a specific interaction between this complex and the ubiquitinated substrate on the membrane surface [106]. ESCRT-I and ESCRT-II are then recruited by ESCRT-0. ESCRT-I comprises Vps23, Vps28, Vps37, and Mvb12 (Tsg101, Vps28, Vps37, and hMvb12 in humans) [107, 108] and may recognize cargo molecules via a ubiquitin-binding domain on Vps23 [108]. ESCRT-II consists of Vps22, Vps36, and Vps25 (human EAP45, EAP30, and EAP20). ESCRT-I and ESCRT-II cooperate to begin vesicle budding [109]. These two protein complexes also recruit ESCRT-III to the budding vesicle [109]. ESCRT-II is formed by Vps20, Snf7, Vps24, and Vps3 (human CHMP6, CHMP4, CHMP3, and CHMP2). ESCRT-0, I, and II are all responsible for the activation of ESCRT-III [110], which mediates the deubiquitination of the cargo with the help of Doa4 (a deubiquitination protein) [111] and vesicle scission [112]. The Vps4-Vta1 complex, which helps dissociate the ESCRT complex [112]. The formed MVBs then fuse with lysosomes/vacuoles and are degraded.

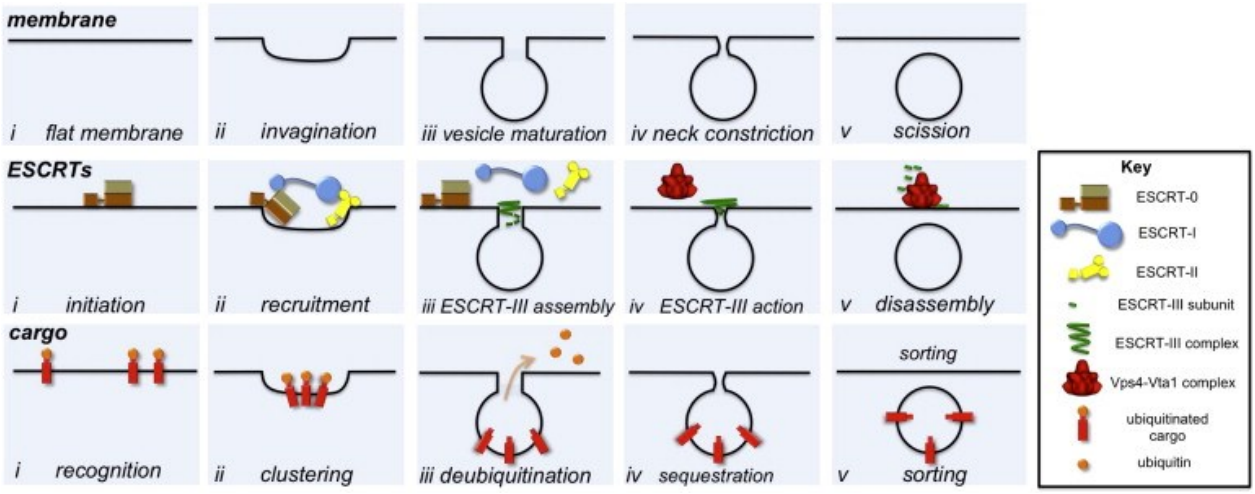

Figure 6 – Overview of the ESCRT pathway. Adapted from [106]. **(i) –** The endosome membrane containing ubiquitinated cargo (depicted as red pins with orange circles as ubiquitin molecules) is first recognized by the ESCRT-0 complex. **(ii)** – This then recruits ESCRT-I which in turn recruits ESCRT-II, which begins the membrane invagination. **(iii)** – In the next step, ESCRT-III is assembled, which leads to vesicle maturation. The cargo is simultaneously deubiquitinated. **(iv)** – The active ESCRT-III complex then constrict the neck of the forming vesicle. **(v)** – Finally, the Vps4-Vta1 complex is recruited, the vesicle is freed from the membrane and the ESCRT machinery is dissembled. For references please see text.

6. Plasma membrane quality control (PMQC)

The final destination proteins in the secretory pathway can reach is the plasma membrane. Therefore, even it has its own quality control mechanism, though very little is known about it. Misfolded PM proteins are degraded via endosomal pathways. For a schematic overview of PMQC pathways please see *Figure 7*.

6.1. Chaperone-mediated PMQC

In mammals, the main quality control mechanism in the plasma membrane is chaperone-mediated. Substrates at the PM may be recognized by a complex of Hsc70 and DNAJA1 along with Hsp90, which then signal to the ubiquitination complex comprising the CHIP E3 ubiquitin ligase [113, 114]. CHIP possesses domains to recruit E2 ubiquitin ligases such as UbcH5c or Ubc13, which interacts with CHIP as a Ubc13-Uev1a complex and mediates K63 polyubiquitination. CHIP is also capable of forming dimers in the cell, though this causes other domains to overlap and blocks the formation of some intermolecular interactions [115]. The ubiquitinated substrate is then endocytosed and marked for lysosomal degradation by the ESCRT pathway as described above. In yeast, such a pathway has not yet been described.

6.2. Ubiquitin ligase-mediated PMQC

The ubiquitin-ligase mediated pathway differs from the above-described mechanism in the way degradation substrates are recognized. While substrates of chaperone-mediated PMQC are first targeted by Hsc70, a chaperone, ubiquitin ligase-mediated PMQC are first targeted by Art1, a ubiquitin ligase adaptor, which signals to Rsp5, a ubiquitin ligase [116]. The substrates are again endocytosed and enter the MVB pathway for degradation.

It is also possible for PM proteins to be endocytosed independently of this mechanism. Those are then subject to quality control via another Rsp5-based mechanism, which targets substrates through Ear1. This protein then recruits Rsp5 for ubiquitination. Misfolded proteins that reach the vacuole unubiquitinated are targeted by Ssh4-Rsp5, which prevents them from accumulating on the vacuolar membrane. Ssh4 and Ear1 are both membrane proteins and are organelle-specific [117].

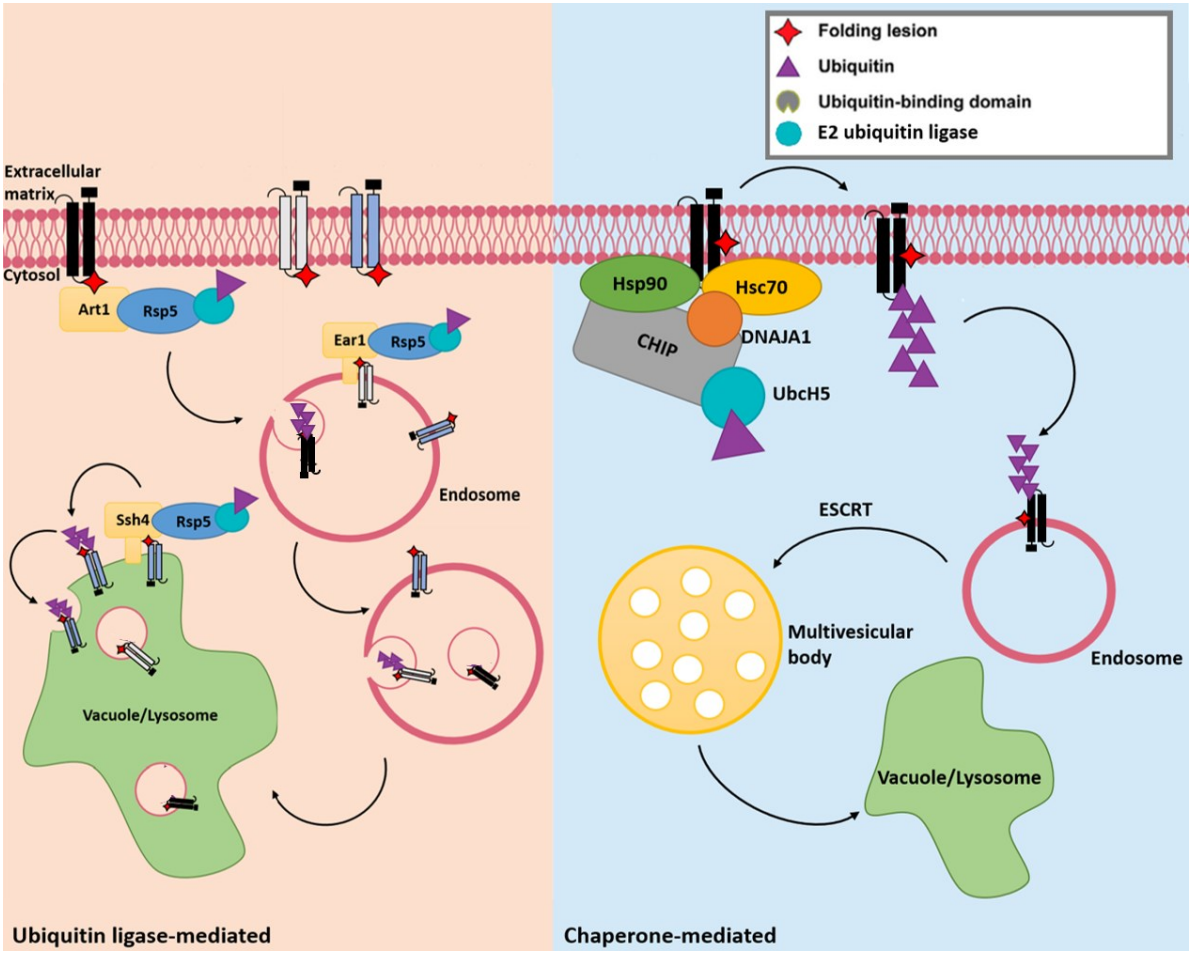

Figure 7 – Overview of PMQC pathways. Adapted from [92]. PMQC is mediated by two specific mechanisms – ubiquitin ligase-mediated degradation and chaperone-mediated degradation. During **ubiquitin ligase-mediated** degradation, protein lesions (shown as red stars) are recognized by ubiquitin ligase adaptors (Art1 on the plasma membrane, Ear1 on endosomal membranes and Ssh4 on vacuolar membranes). These then recruit Rsp5 and an E2 ubiquitin ligase, which ubiquitinate the substrates and lead to endocytosis and degradation in the vacuole/lysosome. **Chaperone-mediated** degradation recognizes proteins via a chaperone complex containing Hsp90, Hsp70 and others. These then mediate the recruitment of a CHIP E3 ubiquitin ligase and an E2 ubiquitin ligase. These ubiquitinate the substrate, which is then endocytosed and formed to an MVB via the ESCRT pathway. This is then degraded via a lysosome or vacuole. For references please see text.

7. Unfolded protein response (UPR)

All the above-mentioned pathways are integrated and balanced by the unfolded protein response (UPR). This is an important mechanism, which comes into play when the quality control mechanisms of the cell are unable to maintain proteostasis due to an upswing in misfolded proteins or protein aggregates. It helps slow protein translation and enlarge the capacity of the quality control pathways.

As shown by Travers et al., the UPR targets many proteins included in the secretory pathway [118]. It upregulates genes for the translocon components, ERAD ubiquitin ligases, and glycosylation related proteins as well as members of ER-phagy pathways or ER-to-Golgi transfer proteins [118]. This strengthens the cell's ability to rid itself of misfolded, aggregated, or overproduced proteins. Also, as discussed at the offset of this thesis, one of the first and rate-limiting steps in ERAD is the mannosylation of the conserved glycan molecule. UPR also upregulates the transcription of α-mannosidase, which could speed this step up and enhance the ERAD pathway further, although not as specifically [118]. The UPR is initiated by three

integral membrane proteins of the ER – IRE1, PERK, and ATF6 – which react to cellular or ER stress. For a schematic overview of UPR pathways please see Figure 8.

7.1. IRE1

IRE1 is one of the most conserved UPR signaling molecules appearing in organisms from yeast (IRE1) to mammals (IRE1α/β). In an inactive state, the luminal domain IRE1 is associated with a molecule of BiP via BiP's ATPase domain. If the level of misfolded or unfolded proteins in the ER rises, they can interact with the free canonical substrate-binding domain (CBD) of BiP and cause the dissociation of BiP from IRE1 [119]. This allows the dimerization (or oligomerization) of IRE1 molecules, which in turn leads to trans autophosphorylation and rearrangement of each monomer and potentiation of the RNase activity of the dimers [120].

The main function of the active RNase is then to splice unspliced *XBP1* (*XBP1u*, *HAC1* in yeast) mRNA molecules, to form an active XBP1 transcription factor. *XBP1u* is transported to the ER as a paused translation intermediate. During translation, the ribosome nascent chain causes the translation of the protein to stop. The hydrophobic N-terminal domain is then exposed for the association of the signal recognition particle (SRP) and the whole complex is recruited to the ER [121]. Here it undergoes splicing, resulting in *XBP1* mRNA for a functional transcription factor. XBP1 regulates the transcription of genes involved in ERAD, proteolysis, protein folding, and trafficking, and many others [122]. Apart from the splicing of *XBP1u*, IRE1 also displays a minimally specific RNase activity dubbed RIDD, by which it degrades ER-associated mRNA molecules [123]. This lowers the influx of proteins into the ER and thus helps ER degradation pathways from being saturated.

7.2. ATF6

ATF6 is an integral membrane protein of the ER present only in mammals. As with IRE1, ATF6 is associated with a BiP molecule in its inactive state [124]. The dissociation of BiP following ER stress allows ATF6 to be trafficked to the GA via COPII vesicles [125]. Here ATF6 is spliced by the serine protease S1P and the intramembrane metalloprotease S2P [126] to give a cytosolically soluble ATF6 domain, which is transported to the nucleus to act as a transcription factor. ATF6 then upregulates the transcription of XBP1 [127] as well as BiP [128].

7.3. PERK

The final molecule I would like to discuss in connection to UPR is PERK. This molecule seems to share many similar mechanisms of activation to IRE1 – it interacts with BiP molecules when inactive and its activation is dependent on the oligomerization and autophosphorylation of PERK monomers [124]. The activation of PERK oligomers leads to the phosphorylation of the eukaryotic initiation factor eIF2α [129]. This halts the translation of proteins which would otherwise burden the ER. It has also been suggested that the PERK/eIF2α interaction might play a role in the quicker formation of autophagosomes [130]. The phosphorylation and subsequent deactivation of eIF2α does, however, have an enhancing effect on the

translation of some proteins such as ATF4 [131]. The translation of this transcription factor causes the upregulation of CHOP, another transcription factor functioning in apoptosis. This in turn activates the expression of GADD34, which dephosphorylates eIF2α and provides a negative feedback loop to this mechanism [132, 133]. Another protein activated by CHOP is ERO1α, an ER oxidase whose prolonged activity can lead to apoptosis [134]. This shows that in a small measure, PERK restores proteostasis to the cell, but if its activation is prolonged, it may also lead to cell death.

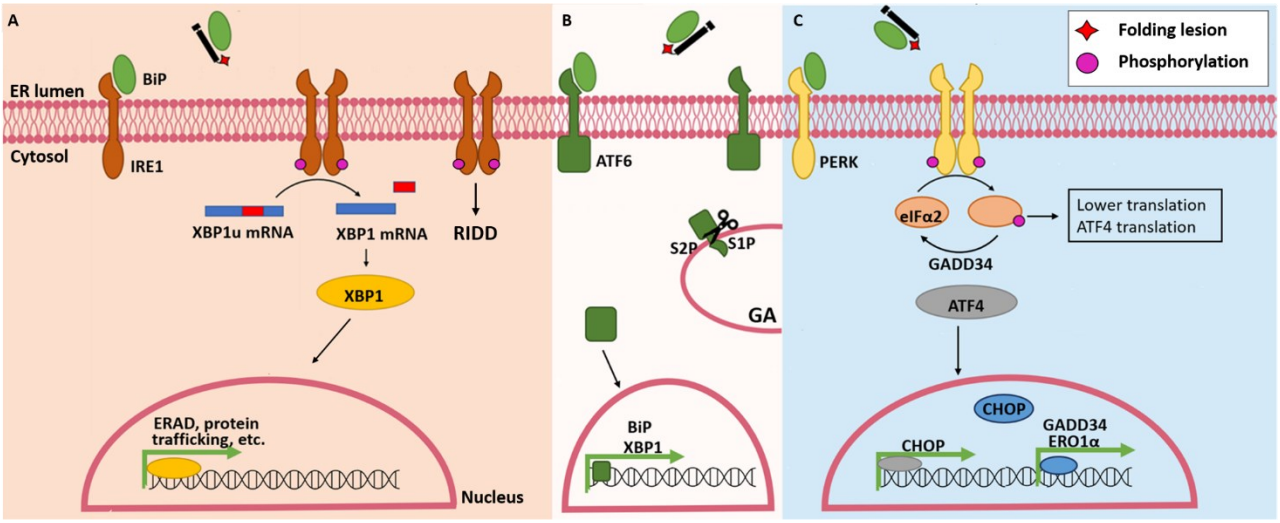

Figure 8 – Overview of UPR pathways in eukaryotic cells. Adapted from [92] and [144]. Three main molecules are involved in UPR pathways – IRE1, ATF6 and PERK. **(A)** – IRE1 is a transmembrane molecule, which interacts with BiP in its inactive state. Upon ER stress or the upswing in the amount of misfolded or aggregated proteins, BiP is dissociated from IRE1, which forms dimers or oligomers. This leads to the trans autophosphorylation (depicted by purple circles) of these molecules and the potentiation their RNase activity. IRE1 can then splice XBPu mRNA (exon shown as a blue rectangle, intron shown as a red rectangle) to give rise to the mRNA for a functional XBP1 transcription factor. XBP1 then enters the nucleus, where it initiates the transcription of genes functioning in the ERAD pathway, protein trafficking, etc. Phosphorylated IRE1 dimers also display a RNase activity called RIDD by which it degrades mRNA molecules bound to the ER membrane. **(B)** – ATF6 is a UPR molecule specific for mammalian cells. As with IRE1, it is activated via the dissociation of a BiP molecule. This allows ATF6 to be transported to the GA, where it is spliced. Its cytosolic domain is released into the cytosol and travels to the nucleus, where it acts as a transcription factor for BiP and XBP1. **(C)** – PERK is activated in a similar manner as IRE1. After auto transphosphorylation, dimers of PERK phosphorylate eIFα2, an initiation factor for translation. This lowers the activity of eIFα2 and leads to lower translation in general. However, the translation of ATF4 is enhanced by a phosphorylated eIFα2. ATF4 then enters the nucleus, where it acts as a transcription factor for another transcription factor (CHOP). This then enables the transcription of GAD34 and ERO1α. For references please see text.

8. Disease relevance

As protein quality control is such a fundamental cellular mechanism, it follows that its defects may have an adverse effect on cellular health. Several diseases have been described to originate from errors in quality control mechamisms.

8.1. Cystic Fibrosis

One of the well-known ERAD substrates in humans in the chloride channel dubbed CFTR (cystic fibrosis transmembrane conductance regulator). The cause of cystic fibrosis is primarily a mutation in the CFTR gene, the most common of which is the ΔF508 deletion, e.g. the single phenylalanine deletion [135]. This class 2 (protein processing) mutation leads to the translation of a temperature-sensitive protein rapidly degraded by the ERAD machinery [136]. It has, however, been shown that the mere lowering of temperature leads to the appearance of functional CFTR channels on the plasma membrane [137]. This shows that despite the present mutation, the protein still has the capacity to form a functional chloride channel and alleviate the

symptoms of cystic fibrosis. Though the lowering of patients' temperatures below 30 °C is not a sustainable therapeutic procedure, the fact that the mutated protein can still function can give us hope of restoring a working channel.

Misfolded CFTR proteins are degraded by the mammalian ERAD pathway. The most important factors for its degradation are Derlin-1 as one of the recognition particles, p97 as a retrotranslocation helper [3], and VIMP as a cofactor aiding in the recruitment of the RNF5 E3 ubiquitin ligase [138]. Studies have shown that the downregulation of various ERAD components such as p97 [139] and VIMP [138] may partially rescue CFTR function. It is possible that this improvement is due to the longer lingering of CFTR mutants in the ER, which enables them to fold properly. Another therapeutic course is the addition of chemical chaperones such as CFcor-325, which help the folding of this protein [140]. The currently used medicament, VX-809, helps to fold the ΔF508 mutant [141], but multistep processes aimed both at the downregulation of ERAD and enhancing the cell's folding capacity could be a more effective therapy in the future.

8.2. Neurodegenerative diseases

Apart from cystic fibrosis, protein quality control can play a key role in a number of neurodegenerative diseases. A decreased level of membralin (a mammalian ERAD component) was shown to increase the number of β-amyloid plaques in patients with Alzheimer's disease. Though the exact mechanism is not yet entirely clear, it seems that membralin is needed for the degradation of the protein nistralin, which otherwise promotes the formation of these plaques [2]. Defects in Parkin (another protein associated with mammalian ERAD) are known to cause Parkinson's disease, though owing to Parkin's many functions, it is unclear whether the cause of Parkinson's disease lies in its absence from the QC pathway or elsewhere. The dysfunctional Parkin is then unable to degrade α-synuclein, whose aggregates cause a rare form of Parkinson's disease [142]. New research has also hypothesized the upregulation of some quality control proteins such as EDEM2 or HRD1 may cause schizophrenia [143].

9. Conclusion

Although the quality control of proteins in the secretory pathway is an integral mechanism for the correct function and viability of cells, there are still many unknowns in the involved pathways. This thesis is aimed at summing up what is already known about the given topic as well as to show where additional information and data is needed. The importance of a well-working protein quality control mechanism is underlined by the fact that several human diseases have been shown to be caused by defects in its mechanism and still more are being unearthed. The knowledge of how this mechanism works could help us cure some of the illnesses which were previously thought to be untreatable. More information is being uncovered all the time, and models that were recently thought to be true may look very different today or in the near future. Even ERAD-L as the most thoroughly described protein quality control pathway is still being reexamined and the model of its mechanism revised. Other quality control pathways require even more research. In my opinion, most information can be obtained from 3D structures of these proteins or protein complexes. Though this was not as good a possibility in the past, novel methods, such as cryo-electron microscopy, are emerging, which could significantly simplify the process and enable us to map these pathways in new ways.

10. Bibliography

Reviews are marker by an asterisk.

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